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(57) Abstract

This invention pertains to vaccine compositions comprising a mixture of antigen, such as an RSV antigen, and the interleukin IL-12, which may be adsorbed onto a mineral in suspension. These vaccine compositions modulate the protective immune response to the antigen.

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VACCINES COMPRISING INTERLEUKIN-12 AND RESPIRATORY SYNCYTIAL VIRAL ANTIGENS

BACKGROUND OF THE INVENTION

The immune system uses many mechanisms for attacking pathogens; however, not all of these mechanisms are necessarily activated after immunization. Protective immunity induced by vaccination is dependent on the capacity of the vaccine to elicit the appropriate immune response to resist or eliminate the pathogen. Depending on the pathogen, this may require a cell-mediated and/or humoral immune response.

The current paradigm for the role of helper T cells in the immune response is that T cells can be separated into subsets on the basis of the cytokines they produce, and that the distinct cytokine profile observed in these cells determines their function. This T cell model includes two major subsets: Th1 cells that produce IL-2 and interferon-γ (IFN-γ) which augment both cellular and humoral immune responses, and Th2 cells that produce IL-4, IL-5 and IL-10 which augment humoral immune responses (Mosmann *et al.*, *J. Immunol. 126*:2348 (1986)). It is often desirable to enhance the immunogenic potency of an antigen in order to obtain a stronger immune response in the organism being immunized and to strengthen host resistance to the antigen bearing agent. A substance that enhances the immunogenicity of an antigen with which it is administered is known as an adjuvant. For example, certain lymphokines have been shown to have adjuvant activity, thereby enhancing the

immune response to an antigen (Nencioni *et_al.*, *J. Immunol. 139*:800-804 (1987); EP285441 to Howard *et_al.*).

SUMMARY OF THE INVENTION

This invention pertains to vaccine compositions comprising a mixture of one or more respiratory syncytial virus (RSV) antigens, interleukin IL-12 and a mineral in suspension. The IL-12 may be either adsorbed onto the mineral suspension or simply mixed therewith. In a particular embodiment of the invention, the IL-12 is adsorbed onto a mineral suspension such as alum (e.g., aluminum hydroxide or aluminum phosphate). These vaccine compositions modulate the protective immune response to the antigen; that is, the vaccine composition is capable of quantitatively and qualitatively improving the vaccinated host's antibody response, and quantitatively increasing cell-mediated immunity for a protective response to a pathogen. In a particular embodiment of the invention, the RSV antigen is an RSV F and/or G protein antigen.

The invention also pertains to methods for preparing a vaccine composition comprising mixing an RSV antigen and IL-12 with a mineral in suspension. In particular, the IL-12 is adsorbed onto the mineral suspension. The invention also pertains to methods for eliciting or increasing a vaccinate's humoral and/or cell-mediated immunity for a protective immune response, comprising administering to a vertebrate host an effective amount of a vaccine composition comprising a mixture of an RSV antigen, IL-12 and a mineral in suspension in a

physiologically acceptable solution. In particular, the IL-12 is adsorbed onto the mineral suspension.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the proliferative responses of splenic immunocytes from BALB/c mice vaccinated with F/AlOH plus ascending doses of IL-12. The dark shaded bars illustrate proliferation after *in vitro* stimulation with native F protein.

Figures 2A and 2B are graphs showing the effect of IL-12 on the ability of F/AlOH to generate IFN-γ (Figure 2A) and IL-5 (Figure 2B) secreting spleen cells in BALB/c mice.

Figure 3 is a graph showing the effects of IL-12 on the capacity of F/AlOH to induce or expand cell-mediated immune responses, and the impact of IL-12 on the capacity of F/AlOH to elicit antigen-dependent killer cells after primary immunization. The solid and dashed lines denote the killer cell activities observed after incubation of bronchoalveolar lavage effector cells with syngeneic RSV-infected or control target cells, respectively, 5 days after challenge.

Figure 4 is a graph showing the effect of IL-12 on the capacity of F/AlOH to boost the cell-mediated immune responses of seropositive BALB/c mice previously infected with RSV. The solid and dashed lines denote the killer cell activities observed after incubation of bronchoalveolar lavage effector cells with syngeneic RSV-infected or control target cells, respectively, 5 days after challenge.

Figure 5 is a graph illustrating the effect of recombinant IL-12 on the protective immune responses induced in BALB/c mice vaccinated with F/AlOH. The bars are one standard deviation of the geometric mean. An asterisk denotes that infectious virus was below detectable levels.

DETAILED DESCRIPTION OF THE INVENTION

IL-12 is produced by a variety of antigen-presenting cells, principally macrophages and monocytes. It is a critical element in the induction of Th1 cells from naive T cells. Production of IL-12 or the ability to respond to it has been shown to be critical in the development of protective Th1-like responses, for example, during parasitic infections, most notably Leishmaniasis (Scott *et al.*, U.S. Patent No. 5,571,515). The effects of IL-12 are mediated by IFN-γ produced by NK cells and T helper cells. IFN-γ is critical for the induction of IgG2a antibodies to T-dependent protein antigens (Finkelman and Holmes, *Annu. Rev. Immunol. 8*:303-33 (1990) and IgG3 responses to T-independent antigens (Snapper *et al.*, *J. Exp. Med. 175*:1367-1371 (1992). Interleukin-12 (IL-12), originally called natural killer cell stimulatory factor, is a heterodimeric cytokine (Kobayashi *et al.*, *J. Exp. Med. 170*:827 (1989). The expression and isolation of IL-12 protein in recombinant host cells is described in International Patent Application WO 90/05147.

The studies described herein relate to the utility of IL-12 as an adjuvant in a respiratory syncytial virus (RSV) vaccine. Accordingly, this invention pertains to

vaccine compositions comprising a mixture of an RSV antigen, IL-12 and a mineral in suspension. In a particular embodiment of the invention, the IL-12 is adsorbed onto a mineral suspension such as alum (e.g., aluminum hydroxide or aluminum phosphate). These vaccine compositions modulate the protective immune response to the antigen; that is, the vaccine composition is capable of eliciting the vaccinated host's cell-mediated immunity for a protective response to the pathogenic antigen. In particular embodiments, the antigen is the RSV F protein and/or G protein.

IL-12 can be obtained from several suitable sources. It can be produced by recombinant DNA methodology; for example, the gene encoding human IL-12 has been cloned and expressed in host systems, permitting the production of large quantities of pure human IL-12. Also useful in the present invention are biologically active subunits or fragments of IL-12. Further, certain T lymphocyte lines produce high levels of IL-12, thus providing a readily available source. Commercial sources of recombinant human and murine IL-12 include Genetics Institute, Inc. (Cambridge, MA). The antigen of this invention, e.g., an RSV antigen, can be used to elicit an immune response to the antigen in a vertebrate such as a mammalian host. For example, the antigen can be an RSV F protein (Collins *et_al.*, *Proc. Natl. Acad. Sci. USA 81*:7683-7687 (1984) or G protein (Satake *et al.*, *Nuc. Acids Res. 13*:7795-7812 (1985) antigen or a portion thereof which retains the ability to stimulate an immune response. Examples of such immunogenic portions are polypeptides comprising amino acid positions 283-315,

289-315 and 294-299 of the RSV F protein. These regions include an epitope of the RSV F protein which elicits both neutralizing and antifusion antibodies (Paradiso *et al.*, U.S. Patent 5,639,853). Alternatively, an RSV F protein in its native dimeric form (140 kD) may be used (Paradiso *et al.*, U.S. Patent 5,223,254).

The method of the present invention comprises administering to a vertebrate an immunologically effective dose of a vaccine composition comprising a mixture of an antigen, e.g., an RSV antigen such as the F and/or G protein, an adjuvant amount of IL-12 and a mineral in suspension. In particular, the IL-12 is adsorbed onto the mineral suspension. As used herein, an "adjuvant amount" of IL-12 is intended to mean a quantity of IL-12 which is sufficient to enhance or modify the immune response to the vaccine antigen, e.g., an RSV antigen such as the F and/or G protein. As used herein, an "immunologically effective" dose of the vaccine composition is a dose which is suitable to elicit an immune response. The particular dosage will depend upon the age, weight and medical condition of the vertebrate to be treated, as well as on the method of administration. Suitable doses will be readily determined by the skilled artisan. The vaccine composition can be optionally administered in a pharmaceutically or physiologically acceptable vehicle, such as physiological saline or ethanol polyols such as glycerol or propylene glycol.

The vaccine composition may optionally comprise additional adjuvants such as vegetable oils or emulsions thereof, surface active substances, e.g., hexadecylamin,

octadecyl amino acid esters, octadecylamine, lysolecithin, dimethyl- dioctadecylammonium bromide, N,N-dicoctadecyl-N'-Nbis (2-hydroxyethyl-propane diamine), methoxyhexadecylglycerol, and pluronic polyols; polyamines, e.g., pyran, dextransulfate, poly IC, carbopol; peptides, e.g., muramyl dipeptide, dimethylglycine, tuftsin; immune stimulating complexes; oil emulsions; lipopolysaccharides such as MPL® (3-O-deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, Montana); and mineral gels. The antigens of this invention can also be incorporated into liposomes, cochleates, biodegradable polymers such as poly-lactide, poly-glycolide and poly-lactide-co-glycolides, or ISCOMS (immunostimulating complexes), and supplementary active ingredients may also be employed. The antigens of the present invention can also be administered in combination with bacterial toxins and their attenuated derivatives. The antigens of the invention can also be administered in combination with other lymphokines, including, but not limited to, IL-2, IL-3, IL-15, IFN-γ and GM-CSF.

The vaccines can be administered to a human or animal by a variety of routes, including, but not limited to, parenteral, intrarterial, intradermal, transdermal (such as by the use of slow release polymers), intramuscular, intraperitoneal, intravenous, subcutaneous, oral and intranasal routes of administration. The amount of antigen employed in such vaccines will vary depending upon the identity of the antigen. Adjustment and manipulation of established dosage ranges used with traditional carrier antigens for

adaptation to the present vaccine is well within the ability of those skilled in the art. The vaccines of the present invention are intended for use in the treatment of both immature and adult warm-blooded animals, and in particular, humans. Typically, the IL-12 and the antigen will be co-administered; however, in some instances the skilled artisan will appreciate that the IL-12 can be administered close in time but prior to or after vaccination with the antigen.

The RSV antigen of the present invention can be coupled to another molecule in order to modulate or enhance the immune response. Suitable carrier proteins include bacterial toxins which are safe for administration to mammals and immunologically effective as carriers. Examples include pertussis, diphtheria, and tetanus toxoids and non-toxic mutant proteins (cross-reacting materials (CRM)), such as the non-toxic variant of diphtheria toxoid, CRM_{197}. Fragments of the native toxins or toxoids, which contain at least one T-cell epitope, are also useful as carriers for antigens. Methods for preparing conjugates of antigens and carrier molecules are well known in the art and can be found, for example, in Dick and Burret, *Contrib Microbial Immunol. 10*:48-114 (Cruse JM, Lewis RE Jr, eds; Based, Krager (1989) and U.S. Patent No. 5,360,897 (Anderson *et al.*).

The adjuvant action of IL-12 has a number of important implications. The adjuvanticity of IL-12 can increase the concentration of protective antibodies produced against the antigen in the vaccinated organism. As a result, effective (i.e., protective) vaccination

can be achieved with a smaller quantity of antigen than would be normally required. This reduction in the required amount of antigen may lead to more widespread use of vaccines which are difficult and costly to prepare. Additionally, the use of IL-12 as an adjuvant can enhance the ability of antigens which are weakly antigenic or poorly immunogenic to elicit an immune response. It may also provide for safer vaccination when the antigen is toxic at the concentration normally required for effective immunization. By reducing the amount of antigen, the risk of toxic reaction is reduced. Furthermore, the adjuvant action can reduce the antigen load of a subject being immunized with a large number of vaccines in a short time.

Typically, vaccination regimens call for the administration of antigen over a period of weeks or months in order to stimulate a "protective" immune response. A protective immune response is an immune response sufficient to protect the immunized organism from productive infection by a particular pathogen or pathogens to which the vaccine is directed. IL-12, when administered with an antigen, such as an RSV antigen including, but not limited to the F protein and the G protein, and mixed with or adsorbed onto a mineral alum in suspension, can accelerate the generation of a protective immune response. This may reduce the time course of effective vaccination regimens. In some instances, it may result in the generation of a protective response in a single dose.

The goal of the work described herein was to determine the feasibility of using recombinant IL-12 as

an immune response modifier for vaccines against RSV. To that end, groups of BALB/c mice were immunized with the native fusion (F) protein of the A2 strain of RSV and ascending amounts of IL-12. The F protein and IL-12 were adsorbed to aluminum hydroxide (AlOH, Alu-gel-S™, Serva Fine Biochemicals, Westbury, NY) adjuvant. Thereafter, the capacities of the vaccines to induce systemic cell-mediated and humoral immune responses were compared. The results presented herein demonstrate that IL-12 is a powerful modifier of both systemic humoral and cell-mediated immune responses. Significant increases in complement-assisted and anti-F protein IgG2a antibody titers were observed after primary and secondary vaccination. In addition, 0.01 and 0.1 μg IL-12 had profound effects on the ability of F/AlOH to elicit cell-mediated immune responses. Five days after challenge with the A2 strain of RSV, the lungs of seronegative and seropositive mice contained augmented antigen-dependent killer cell activities.

To illustrate the mode of action of IL-12 on the immune responses generated by the AlOH adjuvanted vaccines, the supernatants from bulk spleen cells cultured with native F protein were analyzed for the cytokines associated with helper T cell subsets. The results presented herein suggest that IL-12 increases the ability of the vaccines components to elicit immune responses governed by type 1 helper T cells (Th1). The presence of IL-12 in the vaccine appears to be associated with increased amounts of IFN-γ in the culture supernatant. Moreover, the results imply that increased amounts of IL-12 in the formulation diminish

the capacity of the vaccine to generate type 2 helper T cells (Th2). That is, as the amount of IL-12 in the vaccine is increased, the culture supernatants contain lower quantities of IL-5. However at doses of IL-12 above 1.0 µg, both Th1 and Th2 cytokines appear diminished.

Work described herein also examined the immunomodulatory effects of IL-12 on the immunogenicity of RSV native attachment (G) protein and formalin-inactivated RSV (FI-RSV). Both vaccines have been shown in rodents to generate immune responses associated with the induction of Th2 helper T cells. The FI-RSV vaccine was a facsimile of the original Lot-100 vaccine formulated by Pfizer (Fulginiti *et al.*, *Am. J. Epidemiol. 89*:435-448 (1969) and Chin, *et al.*, *Am. J. Epidemiol. 89*:449-463 (1969)). Both vaccines were adsorbed to AlOH and formulated alone or in the presence of 10-fold ascending doses of IL-12.

Several criteria were assessed to determine the immunoregulatory properties of IL-12. The ability of IL-12 to alter the capacity of G/AlOH and FI-RSV to bias recipients for atypical pulmonary inflammatory responses after challenge was particularly significant. Mice twice immunized with either FI-RSV alone or G/AlOH alone, and challenged with the A2 strain of RSV, developed atypical pulmonary inflammatory responses; these inflammatory responses were characterized by statistically significant increased percentages of eosinophils and quantities of IL-5 in the bronchoalveolar lavage (BAL) fluids 5 days after challenge. In contrast, pulmonary eosinophilia was not

observed in control mice experimentally infected with the A2 strain of RSV.

The addition of 10-fold ascending doses of IL-12 to FI-RSV significantly reduced both the relative percentage of eosinophils and the amount of IL-5 detected in the BAL fluids after challenge. Concomitant with the diminution in IL-5 and eosinophils was the significant transformation in serum anti-F and anti-G protein-specific IgG1 to IgG2a antibody. That is, vaccination with FI-RSV containing IL-12 resulted in significant decreases in IgG1 and increases in IgG2a protein-specific antibody titers. The data, therefore, imply that IL-12 limits the induction of F and G protein-specific Th2 helper T cells and their concomitant IL-5 secretion, and, instead, generates IFN-γ- secreting Th1 helper T cells. Thus, the results suggest that the presence of IL-12 in FI-RSV, and ultimately at the site of the immune response, directs the induction of distinct Th1 helper cell subsets. The data also support the idea that the Th1 helper T cells, facilitated by IL-12 injection, hinder the ability of FI-RSV to elicit Th2 helper T cells.

Although the addition of 10-fold ascending doses of IL-12 to G/AlOH resulted in significant reductions in pulmonary IL-5 concentrations and alterations in anti-G and anti-F protein IgG subclass antibody ratios, it appears that IL-12 is unable to modify the capacity of G/AlOH to predispose mice for pulmonary eosinophilia after challenge. Thus, the data suggest that distinct pathways exist for the control of systemic humoral immune responses and the mobilization and replication of

eosinophils. Alternatively, it may be that cytokines other than IL-5 play a role in generating pulmonary eosinophilia. The addition of IL-12 to G/AlOH resulted in significant reductions of IL-5 in the BAL fluids, but had no significant effect on the relative percentage of eosinophils in the pulmonary tissues after challenge. However, IL-5 may be present in the lungs at levels that are non-detectable in the assays employed. It is also possible that IL-12's inability to limit the predilection for eosinophils results from the relatively large amounts of G protein in G/AlOH when compared to FI-RSV, or that native G protein may have several epitopes with potential to bias for eosinophilia. Some of these epitopes may be destroyed by formalin treatment. Thus, the discrepancy between FI-RSV and G/AlOH with respect to their capacity to bias for eosinophilia may be a quantitative phenomenon. The inability of IL-12 to transform the atypical pulmonary inflammatory responses predisposed by G/AlOH, on the other hand, may be related to the uniqueness of the heavily glycosylated protein.

As noted above, the results presented herein demonstrate that IL-12 is a potent regulator of systemic humoral immune responses generated against both purified native G and F proteins of RSV. Nonetheless, the presence of IL-12 does not appear to enhance the complement fixing neutralizing antibody titers attributed to the IgG2a subclass with all RSV vaccines. Augmented serum complement-fixing neutralizing antibody titers were not observed 2 weeks after secondary immunization with either G/AlOH or FI-RSV. With respect

to the FI-RSV vaccine, the failure of IL-12 to positively influence neutralizing antibody titers may reflect the destruction by formalin treatment of the F and G protein epitopes responsible for the generation of IgG2a complement fixing antibodies. As the quantity of IL-12 is increased in the vaccine, neutralizing and IgG1 antibodies are decreased. In contrast, IL-12 reproducibly augments the IgG2a and complement enhanced neutralizing titers in response to F/AlOH (see Tables 1 and 2, below).

The explanation for the inability of IL-12 to influence the generation of IgG2a complement-fixing neutralizing antibodies after vaccination with native G and F protein appears more complex. Without wishing to be bound by theory, one explanation is that the majority of the protein-specific IgG2a antibodies which are elevated following administration of IL-12 are directed against non-neutralizing epitopes. Another possibility is that the contaminating F protein in G/AlOH elicited complement-fixing neutralizing antibodies that obscured the effects of IL-12 on the generation of G protein-specific IgG2a neutralizing antibodies.

The data suggest that the optimal dose of IL-12 is between 0.01 and 1.0 μg; higher doses of IL-12 (10 μg) appear counterproductive. This conclusion is based on the capacity of the vaccine to generate protective humoral and cell-mediated immune responses. Taken together, the results support the use of recombinant IL-12 in vaccine formulations to regulate systemic humoral and cell-mediated immune responses. Furthermore, no untoward effects on the general health of the

recipients were observed after the administration of IL-12.

In summation, the results presented herein show that IL-12 is useful as an immune response modifier for RSV vaccines containing a mineral in suspension. Moreover, the transformation of the immune responses occurs at IL-12 doses appropriate for human use. Thus, it is clear that RSV F and G proteins, alone or in combination with other viral antigens, adjuvanted with an alum gel plus IL-12, are particularly suitable for RSV vaccine preparation.

The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings of all references cited herein are hereby incorporated herein by reference.

EXAMPLES

EXAMPLE 1: Effect of IL-12 on immunogenicity of RSV F protein adsorbed to aluminum hydroxide adjuvant

STUDY DESIGN

The purpose of the study was to determine the effects of recombinant murine IL-12 on the immunogenicity of the fusion protein of RSV formulated with aluminum hydroxide adjuvant. Naive female BALB/c mice (8-10 weeks of age) were vaccinated intramuscularly at weeks 0 and 4 with purified native fusion (F) protein. The F protein was

adsorbed to Alu-Gel-S™ (aluminum hydroxide at 2%, Serva Fine Chemicals, Westbury, NY).

The vaccines were prepared such that each mouse received 3.0 µg F protein/dose, 100 µg of aluminum hydroxide (AlOH) per dose, and 0, 0.01, 0.1, or 1.0 µg IL-12/dose. Control mice were injected with 100 µg Alu-Gel-S™ in PBS alone. Four weeks after primary and 2 weeks after secondary vaccination, serum was collected for the determination by ELISA of geometric mean endpoint antibody titers. The microwells were coated with highly purified ion exchange purified F protein. In addition, neutralizing antibody titers were determined by the plaque reduction neutralization test in the presence and absence of complement against the A2 strain of virus.

To determine the effects of IL-12 on the capacity of the vaccines to generate distinct helper T cell subsets, bulk spleen cell suspensions were obtained 2 weeks after secondary immunization and cultured for 6 days in the presence of increasing concentrations of purified F protein, purified UV-inactivated RSV A2, CRM_{197}, concanavalin A (Con A, a T lymphocyte mitogen), or medium alone. The supernatants from these cultures were pooled and tested by capture-ELISA for the presence of interferon-γ (IFN-γ) and interleukin-5 (IL-5).

RESULTS

The results shown in Table 1 are the geometric mean endpoint antibody titers determined by ELISA. The neutralizing antibody titers are the geometric mean neutralizing antibody titers and were determined by the plaque reduction neutralization test in the presence (+)

and absence (-) of 5% serum to supply complement. The antibody titers were determined 4 and 2 weeks after primary and secondary vaccination, respectively.

IL-12 augmented systemic humoral immune responses generated by F/AlOH 4 weeks (Upper Panel, Table 1) and 2 weeks (Lower Panel, Table 1) after primary and secondary vaccination respectively. For example, 4 weeks after primary immunization with F/AlOH formulated with either 0.1 or 1.0 µg IL-12 per dose, the total IgG endpoint antibody titers were significantly different and enhanced 10 and 18 times, respectively, when compared to the endpoint titers in the sera of mice vaccinated with F/AlOH alone. In similar fashion, the total IgG antibody titers were elevated 3 and 8 times respectively, 2 weeks after secondary vaccination (Lower Panel, Table 1). The data further implied that the increase in IgG antibody titers was dependent on the dose of IL-12 in the vaccine. This was best exemplified after the determination of F protein-specific IgG2a titers. When contrasted with the serum anti-F protein IgG2a antibody titers that were elicited 4 weeks after primary immunization with F/AlOH alone, the vaccines formulated with F/AlOH plus either 0.01, 0.1 or 1.0 µg IL-12 were significantly elevated 4-, 48-, and 158-fold, respectively (Upper Panel, Table 1). Elevations in IgG2a antibody titers were also observed 2 weeks after secondary vaccination with F/AlOH plus IL-12 (Lower Panel, Table 1).

Most importantly, the data suggested that the addition of IL-12 could increase the capacity of F/AlOH to generate complement assisted serum neutralizing antibody titers. The serum complement assisted

neutralizing antibody titers of mice injected with F/AlOH plus 1.0 µg IL-12 were heightened at least 7 times 4 weeks after primary immunization (Upper Panel, Table 1). Following secondary vaccination with F/AlOH formulated with either 0.1 or 1.0 µg IL-12, the neutralizing antibody titers were increased 5 and 10 times, respectively (Lower Panel, Table 1).

The results implied that immune cells from the spleens of mice injected with IL-12 had more potential to replicate when presented with antigen than those of mice vaccinated with aluminum gel alone (Figure 1). For example, the stimulation index of the splenic immunocytes from mice twice immunized with F/AlOH plus 0.01 µg IL-12 was nearly twice that of mice immunized with F/AlOH alone after *in vitro* culture with native F protein. The results further suggested that employing doses of IL-12 greater than 0.01 µg were counterproductive. The stimulation indices of mice vaccinated with F/AlOH plus either 0.1 or 1.0 µg IL-12 were 5-fold less.

To estimate the effect of various doses of IL-12 on the capacity of the aluminum adjuvants to generate distinct antigen dependent helper T cell subsets, bulk spleen cell suspensions were prepared and cultured in the presence or absence of native F protein. The data suggested that when F/AlOH was formulated in the absence of IL-12, type 2-like helper T cell responses were generated. IFN-γ was barely detectable in the supernatants of spleen cells cultured 6 days with native F protein (Figure 2A). Alternatively, the same supernatant contained 6 ng IL-5 per ml culture supernatant (Figure 2B).

The addition of IL-12 to F/AlOH appeared to augment the capacity of splenic immunocytes to secrete IFN-γ (Figure 2A). After 6 days culture with native F protein, 1, 9, 16, and 16 units IFN-γ were secreted per ml supernatant by the spleen cells from mice vaccinated with either F/AlOH alone or plus 0.01, 0.1, or 1.0 µg IL-12, respectively (Figure 2A). However, the data show that the presence of 0.1 or 1.0 µg of IL-12 in the vaccine was associated with decreased amounts of IL-5 in the culture supernatants. Stated otherwise, the same supernatants from mice vaccinated with F/AlOH plus 10-fold ascending doses of IL-12 also secreted 6, 13, 2, and 0.4 ng IL-5 per ml supernatant, respectively (Figure 2B).

Table 1: The ability of recombinant murine IL-12 to modify the systemic humoral immune responses of BALB/c mice immunized with F/AlOH

		ANTIBODY TITERS				
		F Protein (X1000)*			Neutralizing†	
Vaccine	IL-12	IgG	IgG1	IgG2a	(+)	(-)
F/AlOH	1.0 µg	4,621.2	272.9	1,057.4	132[f]	<20
F/AlOH	0.1 µg	2,554.2	263.4	318.9	27	<20
F/AlOH	0.01 µg	586.8[a]	268.9	25.3[a]	22	<20
F/AlOH	NONE	258.3[a]	120.3	6.7[d]	<20	<20
PBS/AlOH	NONE	<0.05	NT	NT	<20	<20
F/AlOH	1.0 µg	12,862.2[b]	889.4	1,730.0	519[g]	55
F/AlOH	0.1 µg	4,524.9	752.7	521.3	224[h]	32
F/AlOH	0.01 µg	3,052.2	1,247.1	205.4[e]	96	22
F/AlOH	NONE	1,521.5[c]	630.4	48.0[d]	49	32
PBS/AlOH	NONE	<1.0	NT	NT	<20	<20

BALB/c mice were vaccinated intramuscularly on weeks 0 and 4 with native F protein (3 µg/dose) adsorbed to aluminum hydroxide (AlOH, 100 µg/dose). IL-12 was added to the vaccines at the indicated doses. Control mice were injected with PBS plus AlOH. The upper and lower panels depict antibody titers 4 and 2 weeks after primary and secondary vaccination respectively.

* The numbers are the geometric mean endpoint antibody titers determined by ELISA.

† The numbers are the geometric mean neutralizing antibody titers and were determined by the plaque reduction neutralization test in the presence (+) or absence (-) of 5% complement. There were 5 mice per group.

[a] $P<0.05$ vs. the IgG titers elicited after vaccination with F/AlOH plus 0.1 or 1.0 µg IL-12.

[b] P<0.05 vs. the IgG titers elicited after vaccination with F/AlOH plus 0.0, 0.01, or 0.1 µg IL-12.

[c] P<0.05 vs. the IgG titers elicited after vaccination with F/AlOH plus 0.1 µg IL-12.

[d] P<0.05 vs. the IgG titers elicited after vaccination with F/AlOH plus 0.01, 0.1, or 1.0 µg IL-12.

[e] P<0.05 vs. the IgG titers elicited after vaccination with F/AlOH plus 1.0 µg IL-12.

[f] P<0.05 vs. the complement dependent neutralizing antibody titers elicited after vaccination with F/AlOH plus 0.0, 0.01, or 0.1 µg IL-12.

[g] P<0.05 vs. the complement dependent neutralizing antibody titers elicited after vaccination with F/AlOH plus 0.0 or 0.01 µg IL-12.

[h] P<0.05 vs. the complement dependent neutralizing antibody titers elicited after vaccination with F/AlOH without IL-12.

EXAMPLE 2: The effect of administration of IL-12 at a distal site on the ability of F/AlOH to induce systemic humoral immune responses.

STUDY DESIGN

The purpose of the study was to determine the biological effects of recombinant murine IL-12 on systemic humoral immune responses when injected at a site distal to that of F/AlOH. To that end, naive female BALB/c mice (8-10 weeks of age) were primed intramuscularly (IM) with ion exchange purified native fusion (F) protein from the A2 strain of RSV. The F protein (3 µg/dose) was adsorbed to aluminum hydroxide adjuvant (AlOH, 100 µg/dose, Alu-gel-S™). F/AlOH was administered in combination with 10-fold ascending doses of recombinant murine IL-12 (1, 10, 100 ng IL-12/dose). In brief, 2 immunization protocols with F/AlOH plus IL-12 were employed. In the first scenario, groups of mice were injected IM with F/AlOH in one thigh and received 10-fold ascending doses of IL-12 in the contralateral thigh. In the second instance, groups of mice were injected once with a vaccine composed of F/AlOH formulated with 1 of 3 10-fold ascending doses of IL-12. The vaccine was incubated overnight at 4°C to allow maximum time for adsorption of IL-12 to AlOH. Additional control mice were vaccinated either with F/AlOH alone, F protein in PBS alone, or PBS plus AlOH alone. Four weeks after primary vaccination, sera were collected for the determination of geometric mean endpoint anti-F protein total and subclass IgG antibody titers by ELISA.

RESULTS

The results depicted in Table 2 confirmed the capacity of IL-12 to augment the systemic humoral immune responses induced after vaccination with F/AlOH. When compared to F/AlOH alone, the anti-F protein total IgG antibody titers elicited by F/AlOH plus either 10 or 100 ng IL-12 were significantly greater 4 weeks after primary immunization. More importantly, the presence of either 10 or 100 ng IL-12 in the vaccines was associated with statistically enhanced protein specific IgG2a antibody titers (Table 2). However, the data also suggested that in a single dose protocol, IL-12 must be present at the local site of injection. When mice were primed with F/AlOH alone and IL-12 was injected at a distal site, statistically lower anti-F protein antibody titers were obtained (Table 2). The protein specific IgG2a antibody titers of mice injected with F/AlOH plus 100 ng IL-12 were 10 times greater than those of cohort mice immunized with F/AlOH alone plus 100 ng IL-12 administered in the contralateral thigh.

Table 2. The effect of distal administration of IL-12 on the ability of F/AlOH to induce systemic humoral immune responses 4 weeks after primary vaccination.†

				ANTIBODY TITERS (LOG 10)		
Vaccine	ng IL-12	#Inj.	#Sites	IgG	IgG1	IgG2a
F/AlOH	100	2	2	5.6±0.2[b]	5.3±0.2[b]	4.7±0.3[b]
F/AlOH	100	1	1	6.9±0.2[a]	6.0±0.1[b]	6.2±0.2[a]
F/AlOH	10	2	2	5.8±0.2[b]	5.5±0.2[b]	5.0±0.3[b]
F/AlOH	10	1	1	6.6±0.3[a]	5.9±0.2[b]	6.0±0.3[a]
F/AlOH	1	2	2	6.0±0.2[b]	5.5±0.2[b]	5.2±0.4[b]
F/AlOH	1	1	1	6.1±0.2[b]	5.6±0.2[b]	5.4±0.3[b]
F/AlOH	None	1	1	6.1±0.2	5.5±0.2	5.2±0.3
F/PBS	None	1	1	3.5±0.5	3.3±0.4	<3.0
PBS	None	1	1	<3.0	NT	NT

† Naive female BALB/c mice were primed intramuscularly (IM) with native fusion (F) protein (3 µg/dose) adsorbed to aluminum hydroxide adjuvant (AlOH). F/AlOH was administered in combination with 10-fold ascending doses of recombinant murine IL-12 (100, 10, 1 ng IL-12/dose). Two immunization strategies with F/AlOH plus IL-12 were employed: 2 inj., 2 sites denotes mice which were injected IM with F/AlOH in one thigh and received 10-fold ascending doses of IL-12 in the contralateral thigh; 1 inj., 1 site indicates that mice were injected once with a vaccine composed of F/AlOH plus 1 of 3 10-fold ascending doses of IL-12. Additional control mice were vaccinated either with F/AlOH alone, F protein in PBS alone, or PBS plus AlOH alone. The numbers are geometric endpoint titers of 5 mice per group.

[a] $P<0.05$ vs. the serum anti-F protein antibody titers from mice injected with IL-12 at a distal site, and the titers of mice immunized with F/AlOH alone.

[b] $P>0.05$ vs. the serum anti-F protein antibody titers from mice immunized with F/AlOH alone.

EXAMPLE 3: The effect of IL-12 on the ability of F/AlOH to induce cell-mediated immune responses in seronegative recipients.

STUDY DESIGN

The purpose of the experiment was to ascertain if the addition of IL-12 to F/AlOH could augment cell-mediated immune responses. Naive female BALB/c mice (8-10 weeks of age) were primed intramuscularly with one of three F protein based vaccines plus recombinant murine IL-12. The native F protein was obtained from the A2 strain of RSV after ion exchange purification. The vaccines were composed of F protein (3 µg/dose) adsorbed to aluminum hydroxide (AlOH, 100 µg/dose) plus one of three 10-fold ascending doses of IL-12 (0.01, 0.1, and 1.0 µg IL-12/dose). The aluminum hydroxide was prepared at Wyeth-Lederle Vaccines and Pediatrics, Pearl River, NY. Control mice were vaccinated with F/AlOH alone, or were intranasally administered mock infected Hep2 cell lysate. Four weeks after primary vaccination the mice were challenged intranasally with RSV A2 (~10^6 PFU) and bronchoalveolar lavage (BAL) was performed 5 days later. The cytolytic capacity of the inflammatory cells was determined directly in a standard 4-hour ^{51}Cr release assay after incubation with syngeneic RSV-infected and control target cells.

RESULTS

The data depicted in Figure 3 demonstrated that IL-12 enhanced the ability of F/AlOH to generate cell-mediated immune responses. The cytolytic activity of the BAL

cells from mice primed with F/AlOH plus 0.01 µg IL-12 (filled triangles) was 39% against syngeneic RSV-infected (solid lines) target cells 5 days after challenge. When the BAL cells from mice primed with F/AlOH alone (filled circles) were incubated with the same syngeneic RSV-infected target cells, cytotoxicity was not detected. The results further implied that the killer cell activity was antigen dependent. The BAL cells from mice vaccinated with F/AlOH plus 0.01 µg IL-12 did not lyse syngeneic control targets (dashed lines) not infected with virus.

The data also suggested that doses of IL-12 greater than 0.01 µg were counterproductive for augmenting cell-mediated immune responses (Figure 3). Primary immunization with F/AlOH plus either 0.1 (inverted filled triangles) or 1.0 (right filled triangle) µg IL-12 resulted in BAL cells 5 days after challenge that, when compared to those of mice primed with F/AlOH plus 0.01 µg IL-12, were 3 and 8 times less cytolytic, respectively.

EXAMPLE 4: The effect of IL-12 on the ability of F/AlOH to induce cell-mediated immune responses in seropositive recipients.

STUDY DESIGN

The goal of the study was to determine the effect of IL-12 on the capacity of F/AlOH to expand cell-mediated immune responses in recipients previously infected with RSV. Naive female BALB/c mice (8-10 weeks of age) were primed by experimental infection with the A2 strain of RSV. Four weeks later, the mice were injected intramuscularly with one of three F protein based vaccines plus recombinant murine IL-12. The vaccines were composed of F protein (3 µg/dose) adsorbed to aluminum hydroxide (AlOH, 100 µg/dose) plus one of three 10-fold ascending doses of IL-12 (0.01, 0.1, and 1.0 µg IL-12/dose). The aluminum hydroxide was prepared at Wyeth-Lederle Vaccines and Pedatrics. Control mice were primed by infection with RSV and secondarily vaccinated with F/AlOH alone, or were intranasally administered mock infected Hep2 cell lysate. Two weeks after secondary vaccination the mice were challenged intranasally with RSV A2 (~10^6 PFU) and bronchoalveolar lavage (BAL) was performed 5 days later. The cytolytic capacity of the inflammatory cells was determined directly in a standard 4-hour ^{51}Cr release assay after incubation with syngeneic RSV-infected and control target cells.

RESULTS

The data depicted in Figure 4 demonstrated that all three doses of IL-12 amplified the ability of F/AlOH to

expand the cell-mediated immune responses of mice previously infected with RSV. The cytolytic activity of the BAL cells from mice secondarily vaccinated with F/AlOH plus 0.1 µg IL-12 (inverted filled triangles) was 58% (effector:target ratio = 54:1) against syngeneic RSV-infected (solid lines) target cells 5 days after challenge. When the BAL cells from mice primed by infection and secondarily vaccinated with F/AlOH alone (filled circles) were incubated with the same syngeneic RSV-infected target cells, cytolytic activity was 35%. When the killer cell activities were examined at the 18:1 effector:target ratio, the cytolytic activities were 52% and 27%, respectively. The results further implied that the killer cell activity was antigen dependent. The BAL cells from mice primed with RSV and secondarily vaccinated with F/AlOH plus IL-12 did not lyse control targets (dashed lines) not infected with virus.

The data further suggested that the ability of F/AlOH to expand cell-mediated immune responses was dependent on the dose of IL-12 in the vaccine (Figure 4). In seropositive animals, the greatest level of antigen-dependent killer cell activity appeared to occur in the lungs of mice secondarily immunized with F/AlOH plus 0.1 µg IL-12 5 days after challenge (inverted filled triangles, Figure 4).

EXAMPLE 5: Effect of IL-12 on Predisposition for Atypical Pulmonary Inflammatory Response

STUDY DESIGN

The purpose of this study was to determine the effect of IL-12 on the capacities of either highly purified native G protein or a facsimile of the Pfizer Lot 100 formalin inactivated vaccine to predispose BALB/c mice for atypical pulmonary inflammatory responses after challenge. Both vaccines were adsorbed to aluminum hydroxide adjuvant.

Naive female BALB/c mice (8-10 weeks of age) were vaccinated intramuscularly (IM) at weeks 0 and 4 with either 1 µg purified native attachment glycoprotein (G) adsorbed to aluminum hydroxide (AlOH, Alu-gel-S™, Serva, 100 µg dose) adjuvant or 0.1 ml formalin-inactivated RSV (FI-RSV). The FI-RSV vaccine was a facsimile of the original Lot-100 vaccine formulated by Pfizer and was adsorbed to AlOH (1600 µg dose). This vaccine was used as a benchmark for atypical pulmonary inflammatory response, an undesirable immune response for subunit vaccines.

IL-12 was added to G/AlOH and FI-RSV in 10-fold ascending doses (0.1 to 10.0 µg IL-12/dose). Additional companion groups of mice were infected (0.05 ml intranasally) with the A2 strain of RSV, injected IM with 0.1 ml formalin-inactivated parainfluenza virus type 3 (FI-PIV3) vaccine adsorbed to AlOH (1600 µg/dose), or were intranasally administered 50 µl of mock-infected Hep2 cell lysate (MOCK). Two weeks after secondary vaccination, serum was collected for the determination of

geometric mean endpoint antibody titers by ELISA in microwells coated with either affinity-purified G protein or ion exchange purified F protein. Geometric mean neutralizing antibody titers were also determined by the plaque reduction neutralization test in the presence and absence of complement against the A2 strain of the virus.

To characterize the effect of IL-12 on the local pulmonary inflammatory responses, bronchoalveolar lavage (BAL) was performed 5 days after challenge with infectious virus (RSV A2, ~10^6 PFU). Inflammatory cell morphology was determined after staining of cytopreps with DIF-QIK® (a reagent which stains leukocytes; Baxter International, Inc., Deerfield, Illinois) and the enumeration of at least 400 cells. The BAL fluids were also examined for the presence of IFN-γ and IL-5 by capture-ELISA. Statistical differences between the groups in IL-5 and IFN-γ secretion were determined after comparing the OD_{490} by ANOVA (analysis of variance by JMP Software; SAS Institute, Cary, North Carolina).

RESULTS

The data suggest that parenteral immunization with the facsimile of the Lot-100 FI-RSV vaccine predisposed BALB/c mice to generate F and G protein-dependent helper T cell responses that were predominantly of the type 2 (Th2) phenotype. The serum anti-G (Table 3) and serum anti-F (Table 4) protein IgG1 to IgG2a antibody ratios 2 weeks after secondary immunization supported this conclusion. The ratio of serum anti-F protein IgG1 to IgG2a antibody titers observed after secondary vaccination with FI-RSV was 75.3 (Table 4). Likewise,

the ratio of serum anti-G protein IgG1 to IgG2a antibody titers observed 2 weeks after secondary vaccination was greater than 184.2 (Table 3).

Moreover, immunization with FI-RSV was associated with an elevation in IL-5 production and the induction of atypical pulmonary inflammatory immune responses in the BAL fluids 5 days after challenge (Table 6).

In Table 6, the results in the "% EOS" column are the geometric mean relative percentage of eosinophils (EOS) enumerated in the BAL fluids 5 days after challenge with virus. "ND" denotes not determined. IL-5 was detected by capture ELISA and quantified from a standard curve. The results in the "IL-5 (OD)" column are the geometric mean optical density (OD_{490}).

The geometric mean relative percentage of eosinophils (38.5%) observed in the lungs of mice vaccinated with FI-RSV 5 days after challenge was significantly elevated when compared with that of control mice vaccinated with FI-PIV3 (6.5%) and undergoing primary infection, or mice immunized by experimental infection (<1.0%) (Table 6). In addition, the amount of IL-5 secreted into the lavage fluids of mice vaccinated with FI-RSV (106 pg/ml) was significantly elevated when contrasted with those fluids from recipients of either FI-PIV3 (<8 pg/ml) or infectious virus (<35 pg/ml) 5 days after infection (Table 6).

The results indicate that the G protein employed in this study contained concentrations of F protein that were immunogenic for BALB/c mice. Noteworthy were the serum anti-F protein total IgG antibody titers observed 2 weeks after secondary vaccination with G/AlOH (Table 4).

The data implied that both G and the contaminating F protein in G/AlOH, like the FI-RSV vaccine, induced primarily Th2 helper T cell subsets. The serum anti-F protein IgG1 to IgG2a antibody ratios after secondary vaccination were greater than 617 (Table 4). In similar fashion, the serum G protein-specific IgG1 to IgG2a antibody ratios after secondary vaccination with G/AlOH were greater than 1251 (Table 3). In addition, immunization with G/AlOH was associated with the induction of atypical pulmonary inflammatory immune responses and the presence of IL-5 in the BAL fluids 5 days after challenge (Table 6). The geometric mean relative percentage of eosinophils (35.0%) observed in the lungs of mice twice vaccinated with G/AlOH 5 days after challenge was significantly elevated when compared with that of control mice vaccinated with FI-PIV3 (6.5%) and undergoing primary infection. Moreover, the amount of IL-5 secreted into the lavage fluids of mice vaccinated with G/AlOH (167 pg/ml) was significantly elevated when contrasted with those fluids from recipients of either mock infected Hep-2 cell lysates (<35 pg/ml), infectious virus (<35 pg/ml), or FI-PIV3 (<8 pg/ml) 5 days after infection (Table 6).

In contrast to vaccination with either FI-RSV or G/AlOH, infection with the wild-type A2 strain of RSV elicited F protein dependent type 1 (Th1) helper T cell responses. The systemic humoral immune responses elicited by the F protein contained in infectious virus was characterized by secondary serum anti-F protein IgG1 to IgG2a antibody ratios less than 1.0 (Table 4). Most importantly, atypical pulmonary inflammatory responses

were not associated with previous RSV infection (Table 6). Pulmonary eosinophilia was not observed in naive mice undergoing primary infection (Table 6).

The results suggest that IL-12 had a profound impact on the humoral immune responses generated after vaccination with either the facsimile vaccine or G/AlOH. The results implied that the dose of IL-12 was important. For example, the data suggested that the addition of either 0.1 or 1.0 µg IL-12 to the F protein contaminated G/AlOH resulted in serum anti-F protein IgG2a antibody titers 2 weeks after secondary immunization that were augmented 4 and 1,300-fold respectively when compared to titers from mice vaccinated with G/AlOH alone (Table 4).

The inhibitory effect of IL-12 was also observed following addition of 10 µg to G/AlOH. The data implied that IL-12 limited the ability of G/AlOH to generate anti-F protein IgG1 antibody titers. When compared to the titers generated by G/AlOH alone, secondary immunization with the G/AlOH plus 10 µg IL-12 generated IgG1 antibody titers that were 5-fold and significantly less (Table 4). The data indicated that 10 µg IL-12 inhibited the capacity of the Lot-100 facsimile vaccine to elicit its characteristic anti-F protein total IgG antibody titers. The serum antibody titers were 17 fold less than those of mice twice immunized with FI-RSV plus 0.1 µg IL-12 (Table 4).

The results shown in Tables 3 and 4 are the geometric mean endpoint antibody titers determined by ELISA. 1/2a is the ratio of geometric mean IgG1 to IgG2a subclass antibody titers. "NT" denotes not tested; "ND" denotes not determined.

The addition of IL-12 did not appear to dramatically alter the magnitude of anti-G protein total IgG antibody titers generated after secondary vaccination with either FI-RSV or G/AlOH (Table 3). However, the presence of IL-12 in the vaccines significantly diminished IgG1 antibody titers, while the IgG2a protein specific antibody titers were significantly elevated (Table 3).

The results imply that IL-12 modified the capacity of the vaccines to induce Th2 and Th1 helper T cell responses. The transformations of serum G and F protein specific IgG1 and IgG2a antibody titers (Tables 3 and 4) support this hypothesis. For example, 2 weeks after secondary immunization the F protein-specific antibody subclass ratio was reduced from 617.1 to 92.1 with the addition of 0.1 µg IL-12 to G/AlOH (Table 4). Moreover, a 10-fold increase in IL-12 from 0.1 to 1.0 µg per dose resulted in a reduction in the F protein-specific antibody subclass ratio to 0.1. However, the data suggested that doses of IL-12 greater than 1.0 µg were counterproductive. For example, the F protein-specific IgG2a antibody subclass titers elicited after secondary vaccination with G/AlOH plus 1.0 µg IL-12 were 1,300 times greater when compared to those generated by G/AlOH alone (Table 4). However, immunization with G/AlOH plus 10 µg IL12 resulted in F protein-specific IgG2a subclass antibody titers that were comparable to those generated after vaccination with G/AlOH plus 1.0 µg IL-12.

The data imply that IL-12 has the ability, via the induction of distinct helper T cell subsets, to modify the infiltration and/or replication of eosinophils in the pulmonary tissues after challenge. This was exemplified

by the effect of IL-12 on the capacity of the vaccines to predispose mice for increased amounts of IL-5 and relative percentages eosinophils in the lungs 5 days after challenge (Table 6). When compared with the BAL fluids from mice vaccinated with FI-RSV alone, the addition of 0.1 or 1.0 µg IL-12 to the FI-RSV vaccine significantly reduced the amount of IL-5 and relative number of eosinophils, respectively (Table 6). The addition of 1.0 µg IL-12 per dose to G/AlOH also resulted in the diminution in IL-5 in the BAL fluids after challenge. However, IL-12 did not appear to have any transforming effect on the capacity of G/AlOH to predispose BALB/c mice to pulmonary eosinophilia after challenge (Table 6). It was noteworthy that atypical pulmonary inflammatory responses were not observed in mice immunized by infection with the A2 strain of virus. Furthermore, IL-5 and eosinophilia were not observed in the lungs of control mice 5 days after primary infection (Table 6). IFN-γ was at or near baseline for all groups examined (data not shown).

The results shown in Table 5 are the geometric mean neutralizing antibody titers. The titers were determined 4 and 2 weeks after primary and secondary vaccination respectively by the plaque reduction neutralization test and in the presence (+) or absence (-) of 5% serum as a source of complement.

Although IL-12 transformed the ratio of F and G protein-specific IgG1 to IgG2a antibody subclasses, there was no statistical augmentation in complement-assisted neutralizing antibody titers 4 and 2 weeks after primary and secondary vaccination respectively (Table 5).

Indeed, the results implied that IL-12 diminished serum neutralizing antibody titers 2 weeks after secondary vaccination. The geometric mean serum neutralizing antibody titers of mice vaccinated with FI-RSV alone were at least 4 times greater than that of recipients of FI-RSV plus 10 µg IL-12. The presence of 10 µg IL-12 in G/AlOH was also associated with a statistically significant 10-fold reduction in complement-independent neutralizing antibodies 2 weeks after secondary vaccination. However, the magnitude of the complement-dependent neutralizing antibody titers elicited by the F protein contaminated G/AlOH were equivalent to that of mice vaccinated by experimental infection (Table 5).

Table 3. The effect of IL-12 on the serum G protein specific IgG subclass antibody titers 2 weeks after secondary vaccination with FI-RSV[a].

ANTI-G PROTEIN ANTIBODY TITER					
VACCINE	**IL-12(μg)**	**IgG**	**IgG1**	**IgG2A**	**1/2a**
FI-RSV	10	8,359[b]	<100	200[e]	<0.5
FI-RSV	1	33,136	162	2,013	0.1
FI-RSV	0.1	67,621	2,774	2,942	0.9
FI-RSV	0	19,568[c]	9,209[d]	<50[f]	>184.2
FI-PIV3	0	592	NT	NT	ND
G/AlOH	10	629,245	32,470	113,378	0.3
G/AlOH	1	846,753	73,028	304,257	0.2
G/AlOH	0.1	575,613	108,976	113,700	1.0
G/AlOH	0	1,183,142	614,243[g]	491[h]	1,251.0
RSV	0	557,743	72,432	106,366	0.7
MOCK	0	<50	NT	NT	ND

Table 3 Legend

[a] The numbers are the geometric mean endpoint antibody titers. The titers were determined by ELISA on sera collected 2 weeks after secondary vaccination. NT and ND denote not tested and not determined respectively. There were 5 mice per group.

[b] P<0.05 vs. total serum IgG antibody titers from mice vaccinated with FI-RSV alone or plus 0.1 or 1.0 µg L-12.

[c] P<0.05 vs. total serum IgG antibody titers from mice vaccinated with FI-RSV plus 0.1 µg IL-12.

[d] P<0.05 vs. serum IgG1 antibody titers from mice vaccinated with FI-RSV plus 1.0 or 10.0 µg IL-12.

[e] P<0.05 vs. serum IgG2a antibody titers from mice vaccinated with FI-RSV plus 0.1 µg IL-12.

[f] P<0.05 vs. serum IgG2a antibody titers from mice vaccinated with FI-RSV plus 0.1 or 1.0 µg IL-12.

[g] P<0.05 vs. serum IgG1 antibody titers from mice vaccinated with G/AlOH plus 10.0 µg IL-12.

[h] P<0.05 vs. serum IgG2a antibody titers from mice vaccinated with G/AlOH plus 0.1, 1.0, or 10.0 µg IL-12.

Table 4. The effect of IL-12 on the serum anti-F protein antibody titers generated 2 weeks after secondary immunization with FI-RSV[a].

	ANTI-F PROTEIN ANTIBODY TITER				
	Vaccine	**IL-12(μg)**	**IgG**	**IgG1**	**IgG2a**	**1/2a**
	FI-RSV	10	58,884[b]	376[d]	21,698[e]	0.2
	FI-RSV	1	601,243	33,274	92,944	0.4
	FI-RSV	0.1	980,212	36,188	201,813	0.2
	FI-RSV	0	250,576[c]	74,814	993[f]	75.3
	FI-PIV-3	0	148	<50	<50	ND
	G/AlOH	10	479,623	37,404[g]	136,748[h]	0.3
	G/AlOH	1	819,426	38,925[g]	410,869[h]	0.1
	G/AlOH	0.1	989,555	106,930	1,161	92.1
	G/AlOH	0	450,308	193,754	314	617.1
	RSV	0	1,808,423	103,954	389,162	0.3
	MOCK	0	<50	NT	NT	ND

Table 4 Legend

[a] The numbers are the geometric mean endpoint antibody titers. The titers were determined by ELISA on sera collected 2 weeks after secondary vaccination. NT and ND denote not tested and not determined respectively. There were 5 mice per group.

[b] P<0.05 vs. total serum IgG antibodies from mice vaccinated with FI-RSV plus 0.1 or 1.0 µg IL-12.

[c] P<0.05 vs. total serum IgG antibodies from mice vaccinated with FI-RSV plus 0.1, 1.0, or 10.0 µg IL-12.

[d] P<0.05 vs. serum IgG1 antibodies from mice vaccinated with FI-RSV alone or plus 0.1 or 1.0 µg IL-12.

[e] P<0.05 vs. serum IgG2a antibodies from mice vaccinated with FI-RSV alone or plus 0.1 µg IL-12.

[f] P<0.05 vs. serum IgG2a antibodies from mice vaccinated with FI-RSV plus 0.1 or 1.0 µg IL-12.

[g] P<0.05 vs. serum IgG1 antibodies from mice vaccinated with G/AlOH alone.

[h] P<0.05 vs. serum IgG2a antibodies from mice vaccinated with G/AlOH alone or plus 0.1 µg IL-12.

Table 5. The effect of IL-12 on the ability of native G protein adsorbed to AlOH and FI-RSV to induce serum neutralizing antibodies.

		NEUTRALIZING ANTIBODY TITERS[a]			
		PRIMARY		SECONDARY	
VACCINE[b]	IL-12(μg)	(+)	(-)	(+)	(-)
FI-RSV	10.0	<10	<10	<20	<20
FI-RSV	1.0	13	<10	34	<20
FI-RSV	0.1	27	<10	90[c]	<20
FI-RSV	NONE	11	<10	85[c]	<20
FI-PIV3	NONE	<10	<10	<20	<20
G/AlOH	10.0	38	<10	1,116	35[d]
G/AlOH	1.0	150	13	2,155	67[d]
G/AlOH	0.1	99	22	1,935	117
G/AlOH	NONE	41	12	3,278	382
RSV	NONE	194	14	2,794	113
MOCK	NONE	<10	<10	<20	<20

[a] The numbers are the geometric mean neutralizing antibody titers. The titers were determined 4 and 2 weeks after primary and secondary vaccination respectively by the plaque reduction neutralization test and in the presence (+) or absence (-) of 5% complement. There were 5 mice per group.

[b] BALB/c mice were immunized intramuscularly with either FI-RSV or G/AlOH alone, or plus 10-fold ascending doses of recombinant murine IL-12.

[c] $P<0.05$ vs. serum neutralizing antibody titers from mice vaccinated with FI-RSV plus 1.0 or 10.0 μg IL-12, or FI-PIV3.

[d] $P<0.05$ vs. serum neutralizing antibody titers from mice vaccinated with G/AlOH alone, 1 μg IL-12 or 10 μg IL-12.

Table 6. The effect of IL-12 on the pulmonary inflammatory responses of BALB/c mice vaccinated with either native G protein adsorbed to AlOH or formalin-inactivated RSV 5 days after challenge with the A2 strain of RSV.

VACCINE[a]	IL-12	%EOS[b]	IL-5 (pg/ml)	IL-5 (OD)[c]
FI-RSV	10	ND	<7	0.029
FI-RSV	1	10.9	<7	0.035
FI-RSV	0.1	17.8	<10	0.044
FI-RSV	0	38.5[d]	106	0.160[f]
FI-PIV3	0	6.5	<8	0.036
G/AlOH	10	31.1	50	0.115
G/AlOH	1	24.7	24	0.074
G/AlOH	0.1	32.8	37	0.096
G/AlOH	0	35.0	167	0.191[g]
RSV	0	<1.0[e]	<35	0.028
MOCK	0	ND	<35	0.035

[a] BALB/c mice were immunized intramuscularly on weeks 0 and 4 with formalin-inactivated RSV (FI-RSV) plus 10 fold ascending doses of recombinant murine IL-12. Control mice were vaccinated with formalin-inactivated parainfluenza virus type 3 (FI-PIV3), natural fusion protein admixed with QS-21 (F/QS-21) or infected with the A2 strain of RSV. Additional control mice received an intranasal administration of mock-infected Hep2 cell lysate (MOCK) or were injected intramuscularly with PBS/QS-21.

[b] The numbers are the geometric mean relative percentage of eosinophils (EOS) enumerated in the BAL fluids 5 days after challenge with virus. ND denotes not determined.

[c] IL-5 was detected by capture ELISA and quantified from a standard curve. The numbers are the geometric mean optical density (OD_{490}).

[d] P<0.05 vs. the eosinophils detected in mice vaccinated with either FI-PIV3 or FI-RSV plus 1.0 µg IL-12.

[e] P<0.05 vs. the eosinophils detected in mice vaccinated with either G/AlOH or FI-RSV alone.

[f] P<0.05 vs. the IL-5 (OD_{490}) detected in mice vaccinated with FI-RSV plus 0.1, 1.0, or 10.0 µg IL-12.

[g] P<0.05 vs. the IL-5 (OD_{490}) detected in mice vaccinated with FI-RSV plus 1.0 µg IL-12.

EXAMPLE 6: The Effect of IL-12 on the Capacity of F/AlOH to Generate Protective Immune Responses in BALB/c Mice.

STUDY DESIGN

The purpose of the study was to investigate the capacity of IL-12 to improve the ability of F/AlOH to generate protective immune responses in the lungs. Naive female BALB/c mice (8-10 weeks of age) were primed intramuscularly (IM) with ion exchange purified natural fusion (F) protein from the A2 strain of RSV. The F protein (30 ng/dose) was administered in combination with PBS alone, or with one of two doses of recombinant murine IL-12 (10 or 100 ng IL-12/dose). The F protein and IL-12 were adsorbed to aluminum hydroxide adjuvant (AlOH, 100 µg/dose) overnight at 4°C. Additional control mice were immunized by experimental infection with the A2 strain of RSV. Four weeks after primary immunization, all mice were challenged with RSV A2 (50 µl, ~5 X 10^6 PFU). The level of virus replication in the pulmonary tissues was assessed four days later. Briefly, the lung and tracheal tissues were removed *en bloc*, homogenized, clarified, snap frozen, and stored at -70°C until assayed for infectious virus. The level of virus replication in the respiratory tract tissues was assessed in a plaque assay employing Hep-2 cell monolayers. Sera were also collected four weeks after primary vaccination for the determination of geometric mean endpoint anti-F protein total and subclass IgG antibody titers by ELISA. Geometric mean serum neutralizing antibody titers were also revealed by the

plaque reduction neutralization test against the A2 strain of virus in the presence or absence of 5% complement.

RESULTS

The results shown in Figure 5 are the geometric mean plaque forming units (PFU) of virus per gram of pulmonary tissue determined four days after challenge. The data depicted in Table 7 are the geometric mean endpoint anti-F protein IgG antibody titers determined by ELISA. The neutralizing antibody titers are the geometric mean neutralizing antibody titers and are determined by the plaque reduction neutralization test in the presence (+) or absence (-) of 5% complement. The antibody titers were determined four weeks after primary vaccination.

The addition of either 10 or 100 ng IL-12 to F/AlOH elicited immune responses that were significantly more efficacious than those generated four weeks after primary immunization with F/AlOH in PBS alone. Infectious virus was not detected in the pulmonary tissues of mice primed with F/AlOH plus either 10 or 100 ng IL-12 and challenged with RSV A2 (Figure 5). In contrast, the lungs of mice primed with either F/AlOH in PBS alone, or PBS/AlOH alone contained greater than 3 $\log_{10}$ PFU virus.

An examination of the sera for F protein-specific endpoint IgG and complement-assisted neutralizing antibody titers suggested that the increased efficacy of the IL-12 formulated vaccines was related to heightened systemic humoral immune responses (Table 7). The mean complement-assisted neutralizing antibody titers generated after immunization with F/AlOH plus 100 ng IL-12 were significantly greater than those of mice primed with

F/AlOH in PBS alone. Nonetheless, immunization with F/AlOH plus 10 ng IL-12 did not result in elevated F protein-specific endpoint IgG and complement-assisted neutralizing antibody titers (Table 7). However, the lungs of these mice inhibited virus replication (Figure 5). Thus, a correlation between IL-12, increased systemic humoral immune responses, and improved efficacy could not be established. In all likelihood, the lack of association was related to the less than optimal 30 ng dose of F protein in the vaccine. Regardless, the addition of as little as 10 ng IL-12 enhanced the ability of F/AlOH to elicit systemic immune responses in BALB/c mice that were significantly more efficacious than those generated after vaccination with F/AlOH in PBS alone.

Table 7. The effect of recombinant murine interleukin-12 on the capacity of fusion (F) protein adsorbed to aluminum hydroxide (AlOH) adjuvant to generate protective humoral immune responses in BALB/c mice

		SERUM ANTIBODY TITERS (Log_{10})‡				
		Anti-F Protein			Neutralizing	
Antigen	IL-12 (ng)	IgG	IgG1	IgG2a	+	-
F/AlOH	NONE	5.7±0.1	5.3±0.2	3.2±1.1	1.5±0.2	<1.3
F/AlOH	10	6.2±0.2[b]	5.7±0.3[b]	4.7±1.3[b]	2.2±0.9[b]	1.3
F/AlOH	100	6.3±0.8[b]	5.7±0.7[b]	5.0±1.7[a]	2.5±0.8[a]	1.3
PBS	NONE	<3.0	<3.0	<3.0	<1.3	<1.3
RSV	NONE	5.6±0.1	4.6±0.2	5.9±0.9	2.3±0.3	1.4

‡ BALB/c mice were primed intramuscularly (IM) with ion exchange purified F protein (30 ng/dose) adsorbed to aluminum hydroxide adjuvant (AlOH, 100 µg/dose) adjuvant. The F/AlOH was administered with PBS alone, or in combination with 100 or 10 ng recombinant murine IL-12/dose. Additional control mice were immunized by experimental infection with the A2 strain of RSV. The numbers are geometric endpoint IgG and neutralizing antibody titers (log_{10}) ±1 standard deviation of the mean of 5 mice per group. The neutralizing antibody titers (log_{10}) were determined in the presence (+) or absence (-) of 5% complement.

[a] $P<0.05$ vs. the serum antibody titers from mice vaccinated with F/AlOH in PBS alone.

[b] $P>0.05$ vs. the serum antibody titers from mice vaccinated with F/AlOH in PBS alone.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the scope of the present invention.

What is claimed is:

1. A vaccine composition comprising a mixture of a respiratory syncytial virus antigen, an adjuvant amount of interleukin-12 and a mineral in suspension, and optionally comprising a physiologically acceptable vehicle.

2. A vaccine composition according to Claim 1, wherein the interleukin-12 is adsorbed onto the mineral suspension.

3. A vaccine composition according to Claim 1, wherein the interleukin-12 is human interleukin-12.

4. A vaccine composition according to Claim 1, wherein the mineral in suspension is an aqueous suspension of alum.

5. A vaccine composition according to Claim 4, wherein the alum is aluminum hydroxide or aluminum phosphate.

6. A vaccine composition according to Claim 1, wherein the respiratory syncytial virus antigen is selected from the group consisting of the RSV F protein, the RSV G protein and combinations thereof.

7. A vaccine composition according to Claim 1, wherein the respiratory syncytial virus antigen is conjugated to a carrier molecule.

8. A vaccine composition according to Claim 7, wherein the carrier molecule is selected from the group consisting of tetanus toxin, diphtheria toxin, pertussis toxin and non-toxic variants thereof.

9. A vaccine composition according to Claim 1, wherein the adjuvant amount of interleukin-12 is from about 0.01 µg to about 1.0 µg.

10. A method of eliciting an immune response to a respiratory syncytial virus antigen, comprising administering to a vertebrate host an effective amount of a vaccine composition comprising a mixture of a respiratory syncytial virus antigen, an adjuvant amount of interleukin-12 and a mineral in suspension, and optionally comprising a physiologically acceptable vehicle.

11. A method according to Claim 10, wherein the interleukin-12 is adsorbed onto the mineral suspension.

12. A method according to Claim 10, wherein the interleukin-12 is human interleukin-12.

13. A method according to Claim 10, wherein the mineral in suspension is an aqueous suspension of alum.

14. A method according to Claim 13, wherein the alum is aluminum hydroxide or aluminum phosphate.

15. A method according to Claim 10, wherein the adjuvant amount of interleukin-12 is from about 0.01 µg to about 1.0 µg.

16. A method according to Claim 10, wherein the respiratory syncytial virus antigen is conjugated to a carrier molecule.

17. A method according to Claim 16, wherein the carrier molecule is selected from the group consisting of tetanus toxin, diphtheria toxin, pertussis toxin and non-toxic variants thereof.

18. A method according to Claim 10, wherein the respiratory syncytial virus antigen is selected from the group consisting of the RSV F protein, the RSV G protein and combinations thereof.

19. An immunogenic composition comprising a mixture of a respiratory syncytial virus antigen, an adjuvant amount of interleukin-12 and a mineral in suspension, and optionally comprising a physiologically acceptable vehicle.

20. An immunologic composition according to Claim 19, wherein the interleukin-12 is adsorbed onto the mineral suspension.

21. An immunogenic composition according to Claim 19, wherein the interleukin-12 is human interleukin-12.

22. An immunogenic composition according to Claim 19, wherein the mineral in suspension is an aqueous suspension of alum.

23. An immunogenic composition according to Claim 22, wherein the alum is aluminum hydroxide or aluminum phosphate.

24. An immunogenic composition according to Claim 19, wherein the respiratory syncytial virus antigen is selected from the group consisting of the RSV F protein, the RSV G protein and combinations thereof.

25. An immunogenic composition according to Claim 19, wherein the respiratory syncytial virus antigen is conjugated to a carrier molecule.

26. An immunogenic composition according to Claim 25, wherein the carrier molecule is selected from the group consisting of tetanus toxin, diphtheria toxin, pertussis toxin and non-toxic variants thereof.

27. An immunogenic composition according to Claim 19, wherein the adjuvant amount of interleukin-12 is from about 0.01 µg to about 1.0 µg.

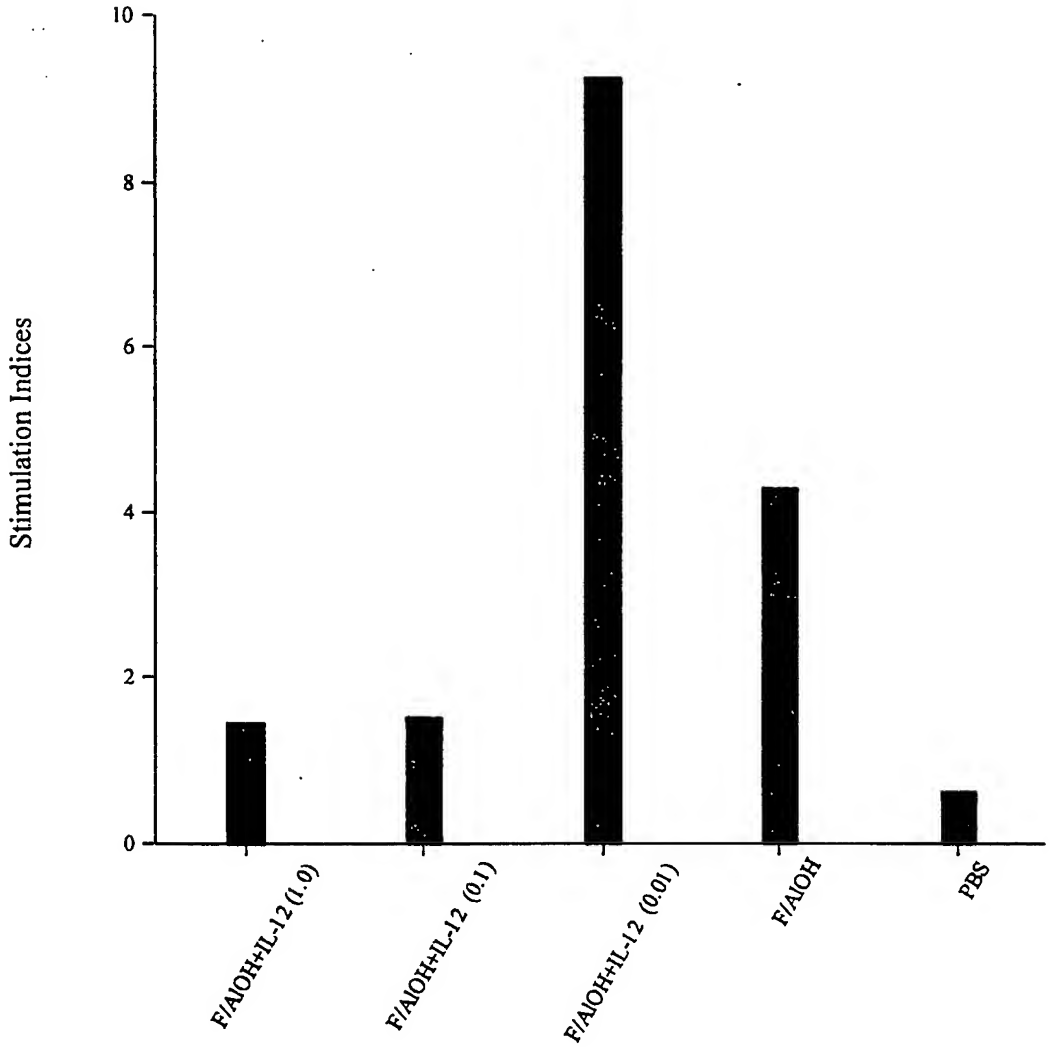

Stimulation Indices
10
8
6
4
2
0
F/AlOH+IL-12 (1.0)
F/AlOH+IL-12 (0.1)
F/AlOH+IL-12 (0.01)
F/AlOH
PBS

Figure 1

Figure 1/2

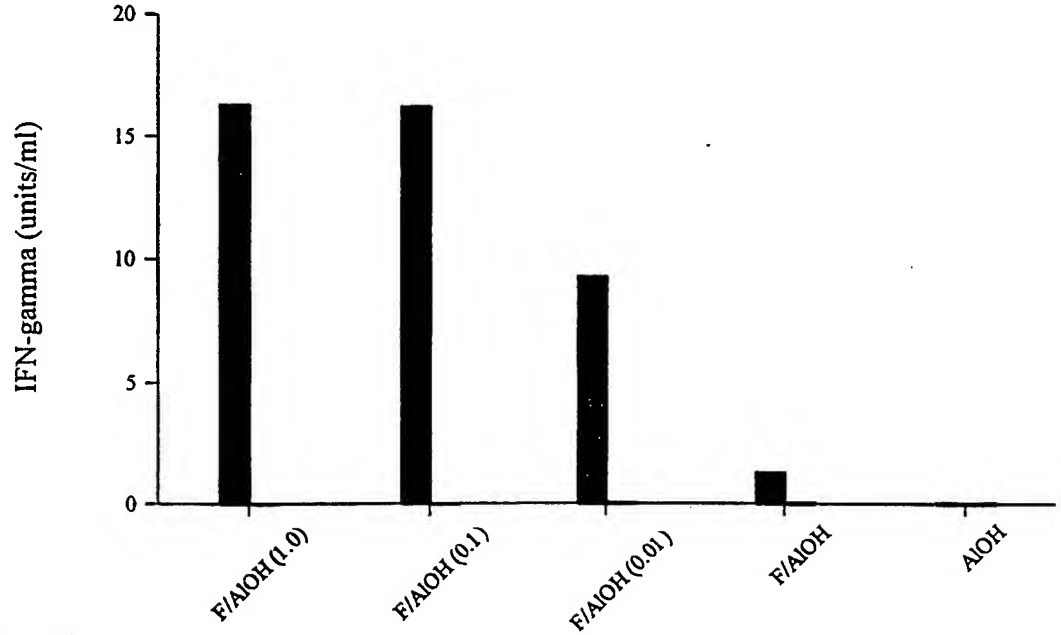
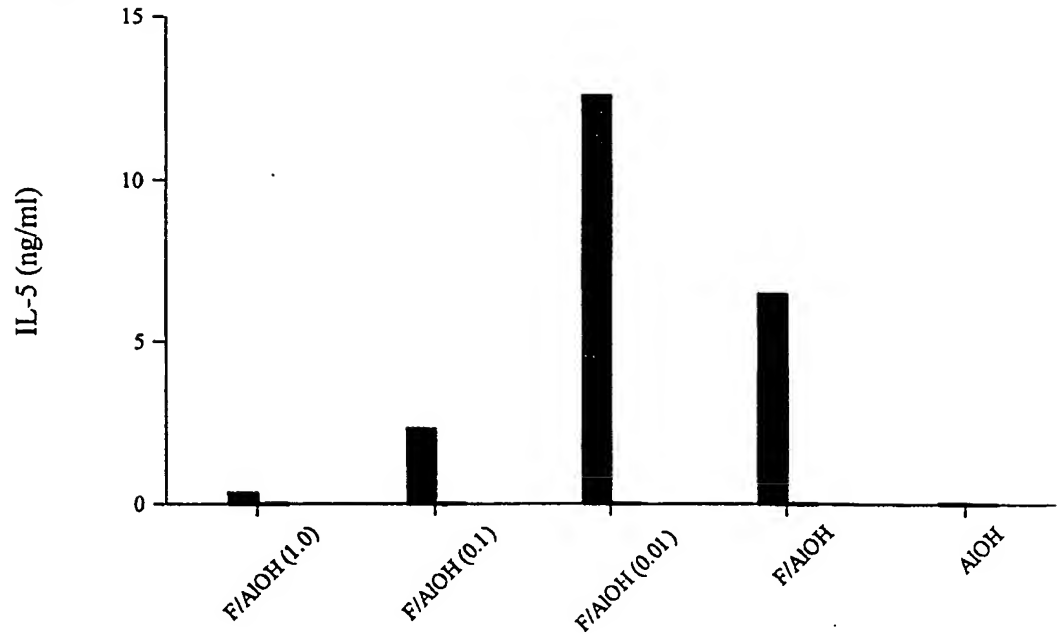


Figure 2/2



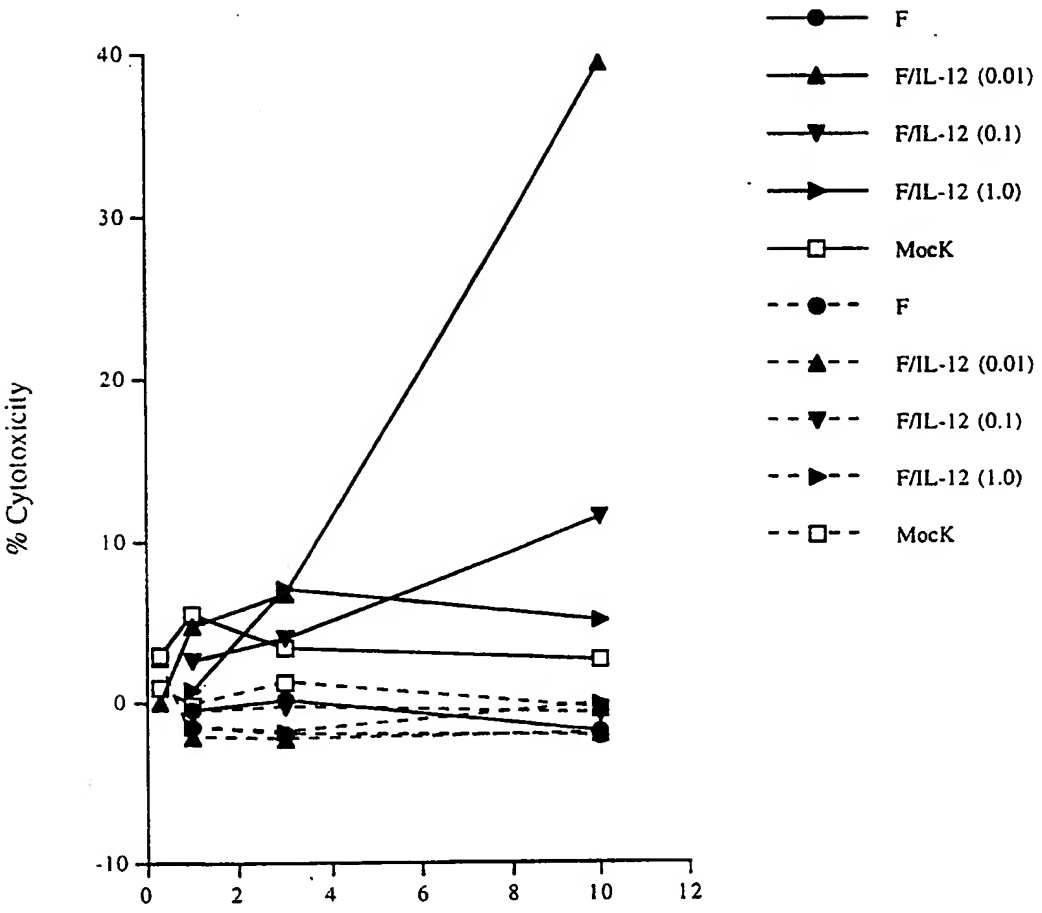

% Cytotoxicity
40
30
20
10
0
-10
0
2
4
6
8
10
12
F
F/IL-12 (0.01)
F/IL-12 (0.1)
F/IL-12 (1.0)
MocK
F
F/IL-12 (0.01)
F/IL-12 (0.1)
F/IL-12 (1.0)
MocK

Figure 3

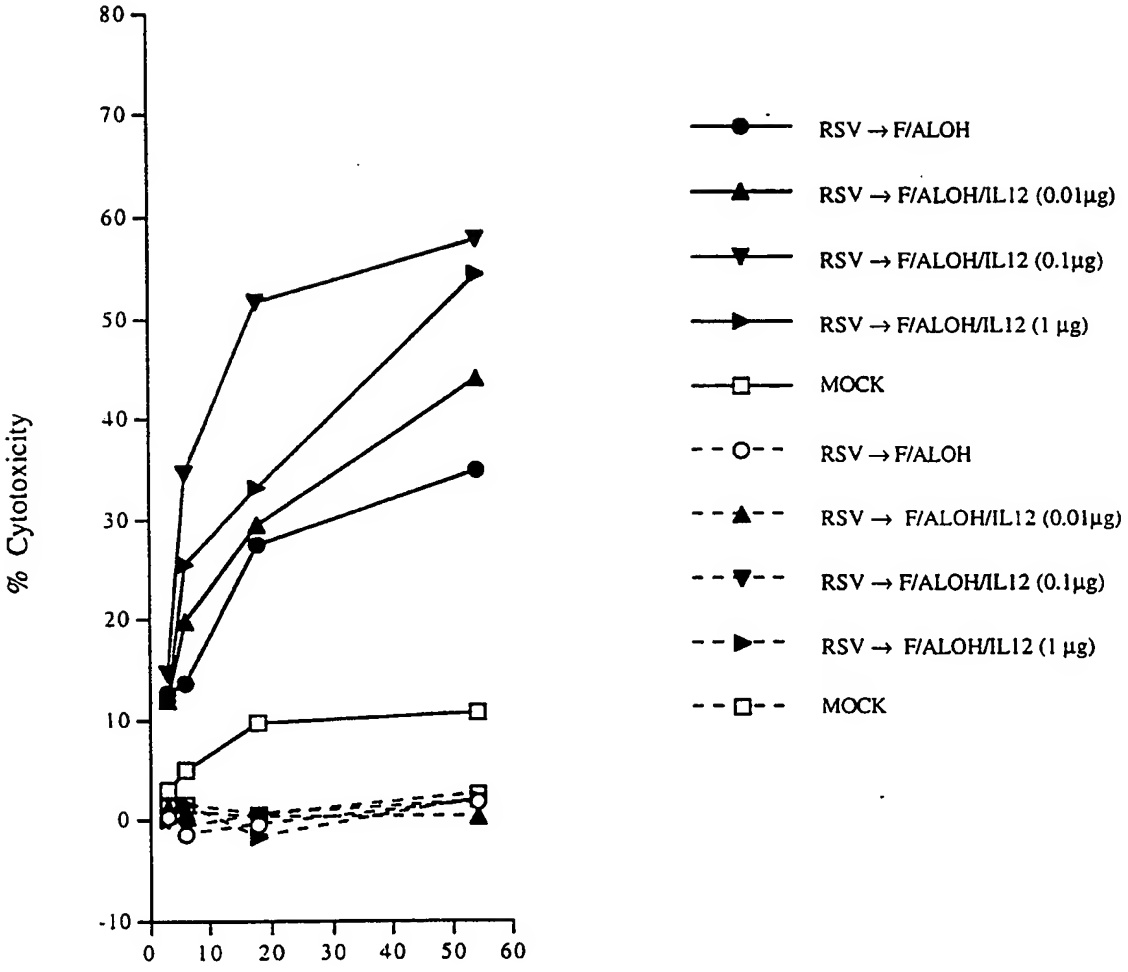

% Cytotoxicity
80
70
60
50
40
30
20
10
0
-10
0
10
20
30
40
50
60
RSV → F/ALOH
RSV → F/ALOH/IL12 (0.01µg)
RSV → F/ALOH/IL12 (0.1µg)
RSV → F/ALOH/IL12 (1 µg)
MOCK
RSV → F/ALOH
RSV → F/ALOH/IL12 (0.01µg)
RSV → F/ALOH/IL12 (0.1µg)
RSV → F/ALOH/IL12 (1 µg)
MOCK

Figure 4

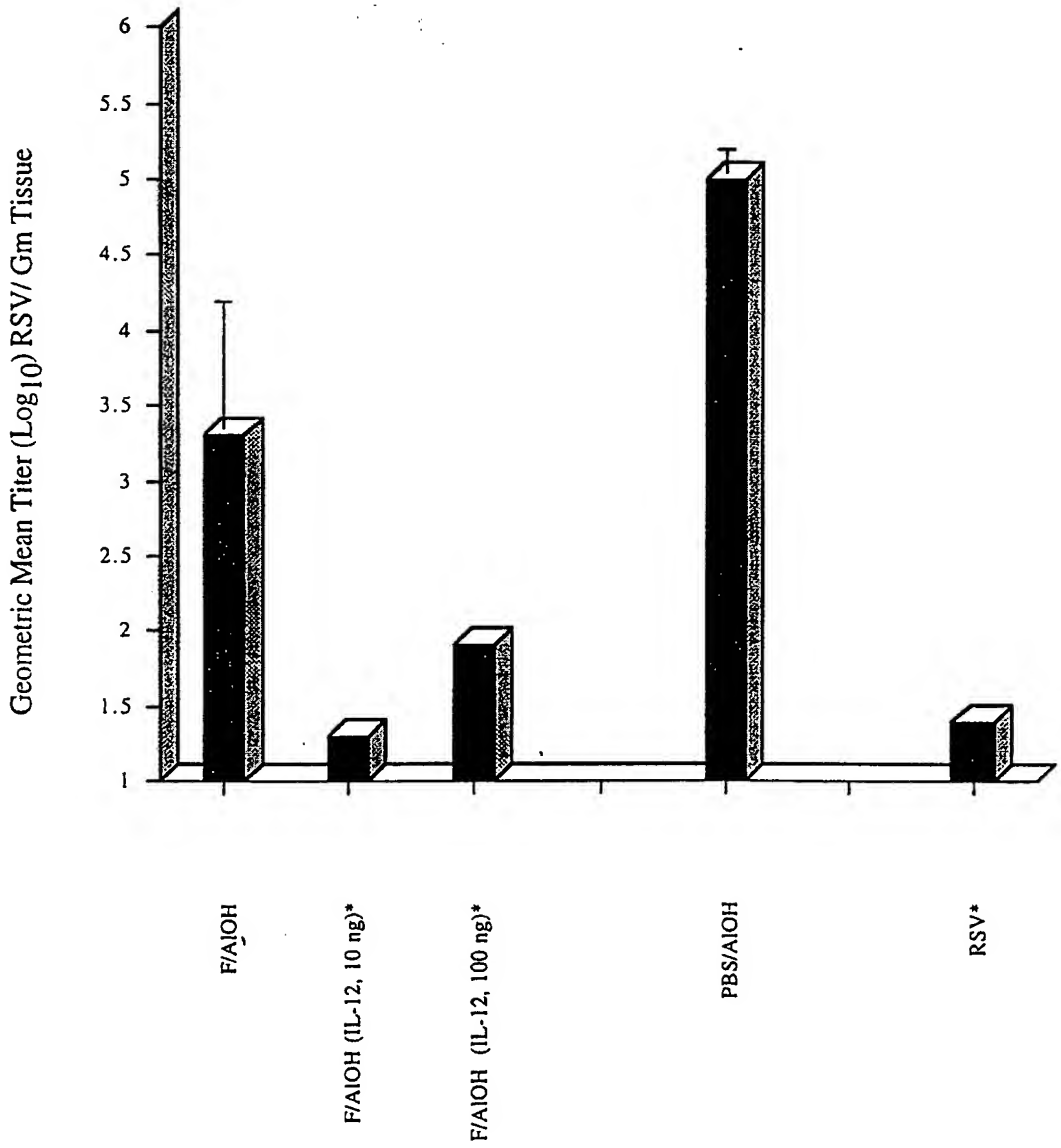

Geometric Mean Titer (Log_{10}) RSV/ Gm Tissue
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
F/AlOH
F/AlOH (IL-12, 10 ng)*
F/AlOH (IL-12, 100 ng)*
PBS/AlOH
RSV*

Figure 5